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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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☐ *Additional inventors are being named on th____ separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

DNA Delivery With Gemini Cationic Surfactants

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ENCLOSED APPLICATION PARTS *(check all that apply)*

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☒ Application Data Sheet. See 37 CFR 1.76
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☒ Other (specify) Sequence listing - 1 page

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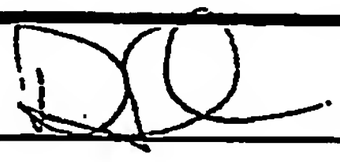
Respectfully submitted,

SIGNATURE 

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Docket Number: 122-03P

TELEPHONE 303-499-8080

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PROVISIONAL PATENT APPLICATION

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DNA DELIVERY WITH GEMINI CATIONIC SURFACTANTS

CERTIFICATE OF MAILING

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DNA DELIVERY WITH GEMINI CATIONIC SURFACTANTS

FIELD OF THE INVENTION

The present invention relates to a delivery system comprising a gemini surfactant with a biologically active agent for treatment of skin disorders and metabolic diseases.

BACKGROUND OF THE INVENTION

As the largest and most accessible organ of the body, skin is an ideal target for gene therapy. Current delivery methods for gene therapy include biolistic or microprojectile introduction, direct injection and electroporation. Although such methods deliver genetic material directly into the skin, they are nevertheless highly invasive. Alternatively, *ex vivo* delivery involves removal of a skin sample from the patient; culturing of skin cells, such as epidermal keratinocytes or dermal fibroblasts; genetically engineering such cells *in vitro*; and returning them in the form of a skin graft back to the patient. However, this procedure may cause unacceptable scarring and trauma to the patient.

Topical delivery of genetic material appears more promising, in that such delivery could provide a more continuous supply of the protein within the skin. This approach has further advantages: i) the DNA is a more stable molecule than the protein, ii) the continuous expression of protein within the skin after topical administration limits systemic exposure; iii) topical treatment could avoid aggravating any lesions by invasive procedures; and iv) topical treatment can be self-administered by the patient. However, these advantages are contingent upon successful delivery of the DNA into the skin.

Gene transfer "vectors" are currently based upon viruses, for which procedures are complex, hazardous, and expensive. Further, repeated dosing is not often possible, and success cannot be guaranteed. Nonviral approaches (e.g., plasmids or oligodeoxynucleotides) are less

expensive, easily manufactured, and can be readily altered to form different combinations depending upon the intended treatment (Vogel, 2000). Further, nonviral approaches permit repeated dosing over time.

Certain types of liposomes have been shown to deliver DNA into the skin tissues both *in vitro* and *in vivo* (Barron *et al.*, 1999; Xu *et al.*, 1999; Birchall *et al.*, 2000; Delepine *et al.*, 2000; Babiuk *et al.*, 2002). Liposomes lack the immunogenicity and hazards associated with viral approaches and allow introduction of larger DNA fragments into target cells. The transfection efficiency is based on physical and chemical characteristics of the building elements of the liposomes.

A gemini surfactant is a surfactant molecule which contains more than one hydrophobic tail. Each hydrophobic tail has a hydrophilic head (Menger and Keiper, 2000; Kirby *et al.*, 2003). The hydrophobic tails or hydrophilic heads are linked together by a spacer. The hydrophobic tails can be identical or differ. Likewise, the hydrophilic heads can be identical or differ. Further, the hydrophilic heads may be anionic (e.g. of a phosphate, sulphate or carboxylate type), cationic (e.g. of a quaternary ammonium type), or neutral (e.g. of a polyether, peptide or sugar type) (Menger and Keiper, 2000). In aqueous solutions, gemini surfactants spontaneously aggregate into micelles whose shape and size are particularly sensitive to the length and hydrophobic or hydrophilic nature of the spacer. The spacer can be variable, namely short (e.g., 2 methylene groups) or long (e.g., more than 12 methylene groups); rigid (e.g., stilbene) or flexible (e.g., methylene chain); and polar (e.g., polyether, ethoxyl, polyethoxyl) or nonpolar (e.g., aliphatic, aromatic) (Menger and Keiper, 2000). As the hydrophobic tails, hydrophilic heads and spacer can vary with regard to the above aspects, innumerable different molecules can be designed. Due to the unique physical properties arising from their structure, gemini surfactants display promise as nonviral delivery systems for biologically active agents. Significantly, topical application of gemini surfactants is most desirable for the advantages previously described. Such a system further provides ease of administration and comfort to the patient, features which are most desirable in gene therapy.

SUMMARY OF THE INVENTION

The present invention broadly relates to a delivery system comprising a gemini surfactant with a biologically active agent in a therapeutically-effective amount for treatment of skin disorders and metabolic diseases.

In another broad aspect, the invention also provides a pharmaceutical composition comprising the delivery system as set out above, in admixture with one or more pharmaceutically acceptable carriers, for the treatment of skin disorders and metabolic diseases.

In another aspect, the invention provides a method of treating skin disorders and metabolic diseases comprising:

contacting the skin or mucosal membrane of a subject with a delivery system comprising a gemini surfactant with a biologically active agent in a therapeutically-effective amount, wherein the delivery system, when in contact with the skin or mucosal membrane, releases the biologically active agent to provide a localized or systemic effect.

In another aspect, the invention provides a delivery system comprising a gemini surfactant with a biologically active agent in a therapeutically-effective amount for treatment of skin disorders and metabolic diseases, wherein the biologically active agent is selected from the group consisting of a nucleic acid, plasmid DNA, DNA vaccine, protein, vaccine, immunoglobulin, immunomodulator, oligonucleotide, peptide, hormone, toxin, and enzyme.

Within a gemini surfactant, the hydrophilic head may be anionic, cationic or neutral, but are preferably cationic in the present invention. In this regard, the present invention broadly relates to a delivery system comprising a gemini cationic surfactant with a biologically active agent in a therapeutically-effective amount for treatment of skin disorders and metabolic diseases.

In another broad aspect, the invention also provides a pharmaceutical composition comprising the delivery system as set out above, in admixture with one or more pharmaceutically acceptable carriers, for the treatment of skin disorders and metabolic diseases.

In another aspect, the invention provides a method of treating skin disorders and metabolic diseases comprising:

contacting the skin or mucosal membrane of a subject with a delivery system comprising a gemini cationic surfactant with a biologically active agent in a therapeutically-effective amount, wherein the delivery system, when in contact with the skin or mucosal membrane, releases the biologically active agent to provide a localized or systemic effect.

In another aspect, the invention provides a delivery system comprising a gemini cationic surfactant with a biologically active agent in a therapeutically-effective amount for treatment of skin disorders and metabolic diseases, wherein the biologically active agent is selected from the group consisting of a nucleic acid, plasmid DNA, DNA vaccine, protein, vaccine, immunoglobulin, immunomodulator, oligonucleotide, peptide, hormone, toxin, and enzyme.

In a further aspect, the invention provides a delivery system comprising a gemini cationic surfactant with a nucleic acid for treatment of skin disorders and metabolic diseases.

In a further aspect, the invention provides a pharmaceutical composition comprising the delivery system as set out above, in admixture with one or more pharmaceutically acceptable carriers, for the treatment of skin disorders and metabolic diseases.

In yet a further aspect, the invention provides a method of treating skin disorders and metabolic diseases comprising:

contacting the skin or mucosal membrane of a subject with a delivery system comprising a gemini cationic surfactant with a nucleic acid, wherein the delivery system, when in contact with the skin or mucosal membrane, releases the nucleic acid to provide a localized or systemic effect.

As used herein and in the claims, the terms and phrases set out below have the meanings which follow:

"Biocompatible" means generating no significant undesirable host response for the intended utility. Most preferably, biocompatible materials are non-toxic for the intended utility. Thus, for human utility, biocompatible is most preferably non-toxic to humans or human tissues.

"Carrier" means a suitable vehicle which is biocompatible and pharmaceutically acceptable, including for instance, one or more solid, semisolid or liquid diluents, excipients, adjuvants, flavours, or encapsulating substances which are suitable for administration.

"Expression" means the transcription of a gene into structural RNA (rRNA, tRNA) or messenger RNA (mRNA) with subsequent translation into a protein.

"Gemini surfactant" means a surfactant molecule which contains more than one hydrophobic tail. Each hydrophobic tail has a hydrophilic head. The hydrophobic tails or hydrophilic heads are linked together by a spacer. The hydrophobic tails can be identical or differ. Likewise, the hydrophilic heads can be identical or differ. Further, the hydrophilic heads may be anionic (e.g. of a phosphate, sulphate or carboxylate type), cationic (e.g. of a quaternary ammonium type), or neutral (e.g. of a polyether, peptide or sugar type), but preferably cationic, and most preferably of a quaternary ammonium type.

"Host" or "host animal" or "subject" means humans or other vertebrates.

"Mucosal membrane" means the epithelial membranes which line the oral cavity, the nasal, bronchial, pulmonary, trachea and pharynx airways, the otic and ophthalmic surfaces, the urogenital system, including the prostate, the reproductive system and the gastrointestinal tract including the colon and rectal surfaces. The term is meant to include the surface membranes or cell structures of the mucosal membrane at a subject's targeted site.

"Pharmaceutically- or therapeutically- effective" means any amount of the delivery system or macromolecule which will exhibit the desired effect upon administration. The amount of the delivery system administered will vary with the condition being treated, the stage of advancement of the condition, the age and type of host, and the type and concentration of the formulation being applied. Appropriate amounts in any given instance will be readily apparent to those skilled in the art or capable of determination by routine experimentation.

"Pharmaceutically- or therapeutically- acceptable" means a substance which does not significantly interfere with the effectiveness or the biological activity of the active agents and which has an acceptable toxic profile for the host to which it is administered.

"Plasmid" means an extrachromosomal hereditary determinant, or a self-replicating circular molecule of DNA which is found in a variety of bacterial, archaeal, fungal, algal, and plant species.

A "polynucleotide" or "nucleic acid" means a linear sequence of deoxyribonucleotides (in DNA) or ribonucleotides (in RNA) in which the 3' carbon of the pentose sugar of one nucleotide is linked to the 5' carbon of the pentose sugar of the adjacent nucleotide via a phosphate group. The "polypeptide" or "nucleic acid" may comprise DNA, including cDNA, genomic DNA, and synthetic DNA, or RNA, which may be double-stranded or single-stranded, and if single-stranded, may be the coding strand or non-coding (anti-sense) strand.

A "protein" or "polypeptide" means a linear polymer of amino acids that are linked by peptide bonds.

"Transfection" means the integration of foreign DNA into the genome of a host cell via direct gene transfer.

A "vector" means a nucleic acid molecule that is able to replicate autonomously in a host cell and can accept foreign DNA.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A shows the general structure of gemini cationic surfactants.

Figure 1B is a schematic illustration of the structure of cationic liposomal formulation (a) and cationic microemulsion (b).

Figure 2 is a graph showing IFNγ expression in PAM212 cells transfected with 0.2μg pIRES.GFP, pIRES.IFN-GFP, pGTmCMV and pGTmCMV.IFN-GFP, using Lipofectamine Plus™ Reagent.

Figure 3 is a color photograph of a fluorescence microscopic evaluation of the GFP expression in PAM 212 cells transfected with (A) 0.2μg pGTmCMV.IFN-GFP plasmid or (B) 0.2μg pGTmCMV plasmid using Lipofectamine Plus™ Reagent.

Figure 4 is a graph showing IFNγ expression in PAM 212 keratinocytes assessed by ELISA, GFP expression and cell viability by FACS.

Figure 5 is a graph showing the influence of transfection duration on the efficiency of the transfection.

Figure 6A illustrates the circular dichroism spectra of plasmid-gemini-DOPE liposomal complexes.

Figure 6B illustrates the circular dichroism spectra of plasmid-gemini complexes.

Figure 7A is a graph showing IFNγ expression in skin in mice treated with the pGTmCMV.IFN-GFP plasmid and gemini lipid 16-3-16 in various formulations.

Figure 7B is a graph showing IFNγ expression in lymph nodes in mice treated with the pGTmCMV.IFN-GFP plasmid and gemini lipid 16-3-16 in various formulations.

Figures 8A-F are color photographs of a fluorescence microscopic evaluation of the GFP expression in skin of mice treated with the pGTmCMV.IFN-GFP plasmid and gemini lipid 16-3-16 in various formulations.

Figures 8A-D show skin obtained from mice topically treated with pGTmCMV.IFN-GFP in cationic gemini liposomal formulation (25 μg in 50μL for three days, total dose 75 μg) (DNA-L-top(3x24h)).

Figure 8E shows skin obtained from mice intradermally injected with pGTmCMV.IFN-GFP in aqueous solution (low concentration of 2.5 μg in 10 μL, total dose 7.5 μg) for one day (DNA-inj-1(24h)).

Figure 8F shows skin obtained from mice topically treated with 50 μL control cationic gemini liposomal formulation (no DNA) for three days (L-top (3x24h)).

DETAILED DESCRIPTION OF THE INVENTION

A. Preparation

Preparation of the delivery system can involve initial preparation of the gemini surfactants with the biologically active agent of interest to form the gemini surfactant-biologically active agent complex, and using the gemini surfactant-biologically active agent complex alone or in combination with supplements to provide formulations for administration to a subject for use in treatment of skin disorders and metabolic diseases. Alternatively, the gemini surfactant does not have to be combined with the biologically active agent first, but can be combined with suitable supplements prior to preparation of the gemini surfactant-biologically active agent complex. The gemini surfactant can thus be combined in any order with the biologically active agent.

i. Gemini surfactants

A gemini surfactant is a surfactant molecule which contains more than one hydrophobic tail. Each hydrophobic tail has a hydrophilic head (Menger and Keiper, 2000; Kirby *et al.*, 2003). The hydrophobic tails or hydrophilic heads are linked together by a spacer. The hydrophobic tails can be identical or differ. Likewise, the hydrophilic heads can be identical or differ. Further, the hydrophilic heads may be anionic (e.g. of a phosphate, sulphate or carboxylate type), cationic (e.g. of a quaternary ammonium type), or neutral (e.g. of a polyether, peptide or sugar type) (Menger and Keiper, 2000). In aqueous solutions, gemini surfactants spontaneously aggregate into micelles whose shape and size are particularly sensitive to the length and hydrophobic or hydrophilic nature of the spacer. The spacer can be variable, namely short (e.g., 2 methylene groups) or long (e.g., more than12 methylene groups); rigid (e.g., stilbene) or flexible (e.g., methylene chain); and polar (e.g., polyether, ethoxyl or polyethoxyl) or nonpolar (e.g., aliphatic, aromatic) (Menger and Keiper, 2000). As the hydrophobic tails, hydrophilic heads and spacer can vary with regard to the above aspects, innumerable different molecules can be designed.

For the present invention, the exemplary type of hydrophobic tail is a C_3-C_{30} alkyl group, linear or branched, saturated or unsaturated. Further, although the hydrophilic heads may be anionic, cationic or neutral, they are preferably cationic in the present invention.

Among the vast classes of gemini surfactants, the types preferable for use in the invention are those with linear hydrocarbon tailgroups and quaternary ammonium headgroups. As illustrated in Figure 1A, the general structure of one type of gemini cationic surfactant is shown to include a head group composed of two positively charged nitrogen atoms, separated by a spacer (n) of 3, 4, 6, 8, 10, 12, or 16 carbon atoms and each containing two methyl groups, and the tails consist of two saturated 12 or 16 carbon atom chains (m =10 or14), respectively. Structures of eight gemini cationic surfactants are further indicated in Figure 1A (Wettig *et al.*, 2001).

Selection of the particular gemini cationic surfactant is significant, in that the magnitude of transfection is significantly dependent on the spacer length of the selected gemini cationic surfactants. A larger Gibbs area (a) per molecule (lower surface excess concentration, Γ, at the air/water interface) (Wettig *et al.*, 2001) correlates with a lower transfection efficiency (see Table 1). These parameters reflect the importance of the spacer length in binding of the gemini cationic surfactant to DNA. The distance between two phosphate groups in a DNA molecule is 0.34 nm, whereas the distance between the cationic head groups in gemini cationic surfactants 12-3-12 and 12-6-12 are estimated to be 0.49 and 0.91 nm, respectively (Chen *et al.*, 2002). Thus, when the distance between head groups in gemini cationic surfactants approaches that between phosphate groups in DNA, stronger complexation may occur.

There is also evidence that longer spacers will bend into a U shape and preferentially locate in a more hydrophobic environment. This U shape formation and the resulting decrease in distance between the two cationic head groups are apparent at spacer lengths greater than twelve carbon atoms (Alami *et al.*, 1993; Chen *et al.*, 2002).

Gemini surfactants can be prepared from readily available starting materials using synthetic chemistry known to those skilled in the art, as reviewed by Menger and Keiper (2000).

ii. Biologically active agents

Biologically active agents which can be used with the present invention include, but are not limited to, nucleic acids, plasmid DNA, DNA vaccines, proteins, vaccines, immunoglobulins, immunomodulators, oligonucleotides, peptides, hormones, toxins, and enzymes.

iii. Supplements

Various supplements can be used to enhance the transfection efficiency. Such supplements generally promote the formation of liposomes around the gemini surfactant-biologically active agent complex. Liposomes are microscopic vesicles containing phospholipid bilayers which enclose aqueous spaces. In a formulation, liposomes carry both water and oil soluble payloads, can solubilize recalcitrant compounds, prevent oxidation, stabilize proteins, and control hydration. Liposomes hold normally immiscible materials together in a microsphere with controllable release of the encapsulated ingredients. For formulations containing the gemini surfactant-biologically active agent complex of the invention, suitable supplements include, but are not limited to:

a) a neutral carrier, such as dioleyl phosphatidylethanolamine (DOPE) which is a nonionic "helper lipid," or cholesterol; and

b) permeation enhancers, for example, Transcutol™ (diethylene glycol monoethyl ether), propylene glycol, oleic acid, and terpenes. There are more than 300 known permeation enhancers, which belong to one of three essential groups based upon their mechanism of permeation enhancement (Williams and Barry, 1992; Chattaraj and Walker, 1995). Group 1 permeation enhancers are capable of extracting skin lipids or damaging the stratum corneum, hence weakening barriers to permeation (e.g., solvents such as ethanol and organic acids such as salicylic acid). Further, Group 2 permeation enhancers increase the solubility of the biologically active agent within the skin (e.g. propylene glycol). Lastly, Group 3 permeation enhancers perturb intercellular lipids (e.g., terpenes, surfactants, fatty acids, fatty acid esters, Azone and derivatives, amides such as dimethylformamide, and sulfoxides such as DMSO).

B. Formation of the Gemini Surfactant-Biologically Active Agent Complex

Gemini surfactants can be prepared from readily available starting materials using synthetic chemistry known to those skilled in the art (Menger and Keiper, 2000).

Biologically active agents (i.e., nucleic acids, plasmid DNA, DNA vaccines, proteins, vaccines, immunoglobulins, immunomodulators, oligonucleotides, peptides, hormones, toxins, and enzymes) can be prepared using techniques known to those skilled in the art before combining with the gemini surfactant to form the gemini surfactant-biologically active agent complex.

In the Examples, the invention is demonstrated using a gene as the biologically active agent. The gene encoding for murine *INFγ* is inserted as part of the plasmid. Briefly, a suitable plasmid is constructed to include the gene encoding the protein of interest, and control sequences such as promoters, enhancers, and terminators, with signal sequences and selectable markers included if desired; for instance, in the Examples, a murine CMV promoter and a *GFP* gene were included for easy qualitative evaluation of protein expression. The *IFNγ* gene was inserted into the multiple cloning site with the *GFP* in a bicistronic format. Such considerations are important, since the level of IFNγ expression was found to be 20 times higher when using the pGTmCMV.IFN-GFP than with the pIRES.IFN-GFP plasmid.

The vector is preferably one which is specifically designed for gene therapy, and is incapable of inducing an immune response; for instance, in the Examples, the vector lacks CpG motifs. The vector should be able to replicate autonomously in a host cell and accept foreign DNA. A vector carries its own origin of replication, one or more unique recognition sites for restriction endonucleases which can be used for the insertion of foreign DNA, and often recognition sequences (e.g. promoter) for the expression of the inserted DNA. Any vector may be used as long as it is replicable and viable in the host.

The gemini surfactant and biologically active agent are combined to form the gemini surfactant-biologically active agent complex using techniques known in the art. In the Examples,

the constructed plasmid is simply mixed with aqueous gemini cationic surfactant to obtain the gemini cationic surfactant-DNA complex.

Preparation of the delivery system can involve initial preparation of the gemini surfactants with the biologically active agent of interest to form the gemini surfactant-biologically active agent complex, and using the gemini surfactant-biologically active agent complex alone or in combination with supplements to provide formulations for administration to a subject for use in treatment of skin disorders and metabolic diseases. Alternatively, the gemini surfactant does not have to be combined with the biologically active agent first, but can be combined with suitable supplements prior to preparation of the gemini surfactant-biologically active agent complex. The gemini surfactant can thus be combined in any order with the biologically active agent.

The gemini cationic surfactant-DNA complex can be used alone or incorporated into topical formulations. Two such formulations are presented in Example 4 and schematically illustrated in Figure 1B. For one formulation (the cationic liposomal formulation), DOPE, DPPC, Transcutol™ and the gemini surfactant, containing a desired concentration of the biologically active agent (e.g., plasmid) is prepared. The supplements assist in formation of liposomes. Formation of the liposome assists in compacting the highly negatively charged DNA into a dense, positively charged or neutral particle small enough to be taken up by the cells. This generally is achieved by use of a highly positively charged compound to neutralize the negative charges of the DNA.

For a second formulation (the cationic microemulsion), the microemulsion is prepared by combining a surfactant, co-surfactant, oily phase component, and the gemini surfactant, containing the biologically active agent (e.g., plasmid) at a desired concentration. PEG-8 caprylic/capric glycerids or other suitable surfactants known in the art can be used. Suitable co-surfactants can include, but are not limited to, polyglyceryl 3- diisostearate, polyglyceryl-6 isostearate, and polyglyceryl-6 dioleate. Suitable oily phase components can be selected from propylene glycol monocaprylate, oleoyl macrogol-6 glycerides, PEG-8 glyceryl linoleate, propylene glycol laurate,

and propylene glycol monolaurate. As an example of a cationic microemulsion, the gemini cationic surfactant-DNA complex is combined with PEG-8 caprylic/capric glycerids as the surfactant; polyglycerol-3-isosterate as the co-surfactant; and octyldodecyl myristate as the oily phase as described in Example 4.

The lipid formulations can be optimized for plasmid DNA:gemini cationic surfactant charge ratio as well known by those skilled in the art. As previously discussed, the magnitude of transfection is significantly dependent on the spacer length of the selected gemini surfactants. A larger Gibbs area (a) per molecule (lower surface excess concentration, Γ, at the air/water interface) (Wettig *et al.*, 2001) correlates with a lower transfection efficiency. These parameters reflect the importance of the spacer length in binding of the gemini cationic surfactant to DNA. The distance between two phosphate groups in a DNA molecule is 0.34 nm, whereas the distance between the cationic head groups in gemini cationic surfactants 12-3-12 and 12-6-12 are estimated to be 0.49 and 0.91 nm, respectively (Chen *et al.*, 2002). Thus, when the distance between head groups in gemini cationic surfactants approaches that between phosphate groups in DNA, stronger complexation may occur.

There is also evidence that longer spacers will bend into a U shape and preferentially locate in a more hydrophobic environment. This U shape formation and the resulting decrease in distance between the two cationic head groups are apparent at spacer lengths greater than twelve carbon atoms (Alami *et al.*, 1993; Chen *et al.*, 2002).

As shown in Example 8, four different plasmid DNA: gemini cationic surfactant charge ratios were tested, and the optimal plasmid DNA: gemini cationic surfactant charge ratio was determined by comparing the quantity of expressed *IFNγ* with the number of fluorescent cells and determining cell viability. In general, a greater than optimal plasmid DNA:gemini cationic surfactant charge ratio results in lower cell viability and lower expression of the protein.

Further, the transfection efficiency of the gemini cationic surfactants can be determined by correlating the physico-chemical characteristics of the gemini cationic surfactants with the expression of the gene of interest. In Example 3, eight gemini cationic surfactants were tested to determine the effect of head group spacer length and alkyl chain length on their transfection efficiency. In this Example, the transfection efficiency was found to be dependent on the length of the spacer between the two positively charged head groups, with the C3 spacer showing the highest activity.

C. Formulations, Dosages, and Treatment

The invention provides a method of delivering biologically active agents by preparing the delivery system (the gemini surfactant-biologically active agent complex as described above) and administering the delivery system topically to the skin or mucosal membrane. Further, the delivery system can be used for localized (intradermal and intramucosal), or systemic (transdermal or transmucosal) delivery, as well as for sustained release in or beneath the skin or mucosal membrane.

For this purpose, the gemini surfactant-biologically active agent complex can be used alone or in any pharmaceutically acceptable vehicle, e.g., lipid formulations, emulsions, creams, lotions, gels, suspensions, aqueous or non-aqueous solutions. Various formulations can be used for administration of the delivery system to the skin or mucosal membrane. Such formulations, whether pharmaceutically acceptable preparations or devices, preferably maintain contact with the skin or mucosal membrane. The delivery system may be incorporated into liquids, gels, creams, lotions, pastes, ointments or foams. Further, substrates such as dressings, packings or meshes can be coated with the delivery system and used directly on the skin or mucosal membrane. Transdermal patches incorporating the delivery system are attached to the skin or mucosal membrane, and provide controlled, sustained release of the biologically active agent in or within the skin or mucosal membrane. As formulations of the delivery system may lose some activity with aging, they can be either stabilized or generated fresh for administration.

The delivery system may be administered alone, or with suitable non-toxic, pharmaceutically acceptable carriers, diluents and excipients suitable for topical application, as are well known in the art.

The dosage of the delivery system depends upon many factors that are well known to those skilled in the art, for example, the particular form of the biologically active agent within the delivery system , the condition being treated, the age, weight, and clinical condition of the recipient patient, and the experience and judgement of the clinician or practitioner administering the therapy. A therapeutically effective amount provides either subjective relief of symptoms or an objectively identifiable improvement as noted by the clinician or other qualified observer. The dosing range varies with the biologically active agent within the delivery system used, its form, the route of administration and the potency of the particular agent.

To demonstrate the preparation and method of use of the delivery system of the present invention, the inventors evaluated the effectiveness of the delivery system in topical delivery of the gene coding for *IFNγ* as potential therapy for treatment of scleroderma. Scleroderma is a complex disease that is classified into two major groups, namely the types that affect the skin only (localized scleroderma: morphoea and linear scleroderma) and types where in addition to skin, internal organs (esophagus, gastrointestinal tract, lungs, kidneys, heart and muscles) are involved (systemic sclerosis: diffuse, limited and other) (Moschella and Hurley,1992). Scleroderma is an autoimmune connective tissue disease in which, for unknown reasons, the skin becomes thick and hard due to the excessive production and deposition of collagen. T-cell derived IFNγ is one of the most potent inhibitors of collagen gene transcription in fibroblasts. *In vitro* studies clearly showed that IFNγ reduces fibroblast collagen synthesis, induces the repression of fibroblast growth and modulates the interactions between cells and intercellular matrix that can lead to a more optimized collagen network (Harrop *et al.*, 1995; Widom, 2000). A model of the molecular pathology of scleroderma shows that IFNγ gene therapy may have an effect on three groups of pathophysiological markers of scleroderma, namely cytokines (1); collagen and extracellular matrix components (2), and cell adhesion molecules (3) (compiled from Bos *et al.*,

1997; Luger *et al.*, 1997; Galperin and Gershwin, 1998; Arnett, 2002). Based on this pathogenesis model, it can be speculated that the administration of IFNγ could also have an indirect inhibitory effect on TGF-β and an immunomodulatory effect on T cells to switch the Th1/Th2 balance toward Th1.

The limitation of the treatment by IFNγ is related to the non-targeted administration method. Subcutaneous or intramuscular injection of IFNγ does not provide sufficient levels of this cytokine within the target tissue; therefore, the main challenge is the delivery and targeting of IFNγ to the skin. The inventors addressed this problem using the delivery system of the present invention. Briefly, a suitable plasmid was constructed. Transfection and cellular expression of IFNγ from pGTmCMV.IFN-GFP plasmid were evaluated in PAM212 keratinocyte culture. The plasmid/gemini cationic surfactant (varying spacer and chain lengths) complexes were characterized by circular dichroism and microscopy. For topical formulations, the inventors prepared two delivery systems, namely a cationic liposomal formulation and a cationic microemulsion, both of which incorporate a gemini cationic surfactant. Mice were treated topically with such formulations and the IFNγ expression was evaluated in the skin biopsies by ELISA.

The *in vitro* transfection efficiency was found to be dependent on the spacer length of the gemini cationic surfactants, with the C3 spacer showing the highest activity (both 12-3-12 and 16-3-16). The gemini cationic liposomal formulation and cationic microemulsion (25μg DNA/dose) produced similarly high levels of IFNγ in the skin (811 vs 934 pg/cm^2) (both statistically different from naïve, $p<0.01$); however, in the pooled lymph nodes, the liposome treated mice showed higher levels of IFNγ (1421 vs 873 pg/animal). IFNγ expression after a single topical application of the plasmid in the gemini cationic liposomal formulation (25μg/dose) was similar to the intradermal injection of naked DNA solution at 15μg DNA/dose. The pGTmCMV.IFN-GFP plasmid was thus found to be functional and effective both *in vitro* and *in vivo*.

The inventors thus found that the delivery system of the present invention demonstrates effectiveness as a topical form of gene therapy. Although the invention is described herein for treatment of scleroderma, it will be appreciated that the invention is equally useful for the therapeutic treatment of other conditions. The delivery system can be used for localized delivery to treat skin disorders, particularly genodermatoses (skin diseases of genetic origin) including epidermal fragility disorders, keratinization disorders, hair disorders, pigmentation disorders, porphyrias, multisystem disorders and cancer disorders, as reviewed by Uitto and Pulkkinen (2000). For example, candidate diseases for treatment with the present invention include, but are not limited to, forms of inherited epidermolysis bullosa (such as junctional EB and dystrophic EB which are characterized by extreme fragility of the skin and mucosal membrane); lamellar ichthyosis and X-linked ichthyosis (characterized by epithelial cornification and defective skin barrier function); and xeroderma pigmentosum (characterized by defective DNA repair functions leading to severe blistering upon exposure to sunlight and development of multiple skin tumours).

Further, the delivery system can be used for systemic delivery to treat metabolic diseases through modification of epidermal keratinocytes within the skin. Epidermal keratinocytes normally secrete factors into the extracellular matrix which then reach the bloodstream (Spirito *et al.*, 2000). Such metabolic diseases which could be amenable to treatment using the delivery system of the present invention include, but are not limited to, gyrate atrophy, maternal hyperphenylalaninemia, familial hypercholesterolemia, and phenylketonuria.

Abbreviations and nomenclature employed herein are standard in the art and are commonly used in scientific publications such as those cited herein.

The invention is further illustrated by the following non-limiting examples.

D. Examples

Example 1 - Preparation of Plasmids

The pGT is a vector designed for gene therapy. It contains the human cytomegalovirus (CMV) promoter, having the CpG motifs removed, where the human CMV was replaced with the

murine CMV (Dorsch-Häsler *et al.*, 1985) to give the pGTmCMV backbone. The *IFNγ* gene (Gray *et al.*, 1983) was obtained from the pSLRSV.IFN plasmid (Lewis *et al.*, 1997) that contained the sequence coding for 155 amino acids of the murine IFNγ. The plasmid pIRES2-EGFP (Clontech, Palo Alto, CA) contains the gene encoding for the enhanced green fluorescent protein fused with IRES sequence, preceded by a multiple cloning site for gene insertion. The gene encoding for murine IFNγ was inserted at the Bgl II site, creating the plasmid pIRES.IFN-GFP. The pGTmCMV.IFN-GFP was constructed by inserting the IFNγ-IRES-GFP fragment into Bgl II and Xba I sites of the pGTmCMV vector. The plasmids were purified using QIAGEN Plasmid Purification Kit (Qiagen, Mississauga, ON). The genes of IFNγ and IFNγ- IRES-GFP were sequenced (PBI/NRC, Saskatoon, SK) and compared with sequences retrieved from GenBank (NCBI databases). Reagents were purchased from Invitrogen Life Technologies (Carlsbad, CA) and restriction enzymes from Amersham Pharmacia Biotech (Baie d'Urfe, QB).

Example 2 - Transfection of PAM212 cells with plasmid constructs, using Lipofectamine Plus™ Reagent

PAM212 murine keratinocyte cells were grown to 90% confluency in 75-cm^2 tissue culture flasks in supplemented MEM, prepared from minimal essential media (MEM) (GIBCO BRL, Burlington ON) with Antibiotic Antimycotic Solution (Sigma, Burlington, ON) 1:100 dilution, and 10%v/v fetal bovine serum (FBS) (Cansera, Etobicoke ON). The day before transfection $5x10^4$ cells/well were seeded on 24-well plates (Greiner Labortechnik GmbH, Germany) on 13-cm diameter cover slips (CANEMCO, St. Laurent, QB). The plates were incubated overnight at 37°C in a CO_2 incubator to 70-80% confluency. The supplemented MEM was changed to MEM one hour prior to transfection. The cells were transfected with the following plasmids: pIRES, pIRES.IFN-GFP, pGTmCMV, and pGTmCMV.IFN-GFP, using Lipofectamine Plus™ Reagent (Invitrogen Life Technologies, Carlsbad, CA). For each well 0.2 μg of plasmid was used. The transfection method followed the manufacturer's protocol and was optimized for the PAM212 cells. Briefly, 0.2 μg of plasmid was mixed with 10 μL of PLUS reagent in 25μL MEM and incubated at room temperature for 15 minutes. Four μL Lipofectamine, mixed with 25μL MEM, was added to the plasmid. After incubating the mixture for 15 minutes at room temperature, it

was added dropwise to cells that were covered with 200 μL of fresh MEM. The plates were incubated for periods of 5 or 6-24 hours at 37°C in a CO_2 incubator (Sanyo Electric Co. Ltd., Japan), then the transfection mix was replaced with supplemented MEM, and the incubation was continued for 24 h, after which the supernatants were collected. Media on the cells was replaced with fresh media, and after another 24 h incubation period, the second supernatants were collected and stored at -20°C.

Example 3 - Transfection of PAM212 cells with pGTmCMV.IFN-GFP plasmid using gemini cationic surfactants and DOPE

The pGTmCMV.IFN-GFP plasmid was used at a concentration of 0.2 μg/well for transfection. Eight gemini cationic surfactants were tested (Figure 1A) (Wettig *et al.*, 2001). Aqueous solutions (1.5mM) of the gemini cationic surfactants were prepared and filtered through 0.2μm Acrodisc® filters (Pall Gelman, Ann Arbor, MI). Lipid vesicles were prepared by using sonication techniques. 1,2 dioleyl-*sn*-glycero-phosphatidylethanolamine (DOPE) (Avanti Polar Lipids, Alabaster, AL) and α-tocopherol (Spectrum, Gardena CA) in 1:0.2 weight ratios were dissolved in 100% ethanol (Commercial Alcohols Inc., Brampton ON) at a concentration of 10 mg lipid/mL in a round bottom flask. The solvent was evaporated in a rotary evaporator (Rotavapor RE111 BÜCHI Laboratoriums-Technik AG, Switzerland) at 100 rpm and 55°C whereupon a thin film was deposited on the walls of the vessel. To remove traces of organic solvent, the lipid was lyophilized overnight. Glass beads were added to the flask and the lipid was resuspended at 1μmol/mL concentration in 9.25 %w/v isotonic sucrose (Spectrum, Gardena, CA) solution (pH 9). The suspensions were bath-sonicated (Branson 2200, Cleansonic Orange, VA) for 3 h at 55°C. The suspensions were filtered through 0.45μm Acrodisc® filters. PAM212 murine keratinocyte cells were prepared for transfection as described earlier.

The transfection mixtures were prepared as follows: 0.2 μg of plasmid was mixed with aliquots of gemini cationic surfactant solution to obtain plasmid DNA: gemini cationic surfactant charge ratios of 1:5, 1:10, 1:20 or 1:40 and incubated at room temperature for 15 minutes. To this mixture, 25 μL of DOPE liposomes were added. The transfection mixtures were incubated

for 30 min at room temperature prior to transfection and added to the cells, dropwise. The plates were incubated for 5 h at 37°C in a CO_2 incubator. The transfection mix was replaced with supplemented MEM and the plates further incubated for 24 hours. The supernatants were collected and stored at -20°C. As a positive control, the cells were transfected with Lipofectamine Plus™ Reagent, as described above.

Example 4 - Topical Formulations

Two topical delivery systems were prepared with the gemini 16-3-16 surfactant. A cationic liposome formulation was prepared with DOPE 10mg/mL, 1.2 dipalmitoyl-*sn*-glycero-phosphatidylcholine (DPPC) (Sigma) 10 mg/mL, the gemini 16-3-16 surfactant 10 mg/mL, and Transcutol™ (Gattefosse, Saint-Priest, France) 25 mg/mL, containing 25 μg of plasmid in 50 μL of formulation (Figure 1B, panel A). A cationic microemulsion was prepared with PEG-8 caprylic/capric glycerids (Gattefosse) 200 mg/mL, polyglyceryl-3-isostearate (Gattefosse) 200 mg/mL, octyldodecyl myristate (Gattefosse) 400 mg/mL and the gemini 16-3-16 surfactant 10mg/mL (Figure 1B, panel B). The plasmid concentration was 25 μg in 50 μL formulation. The formulations were characterized by visual appearance, microscopy and pH.

Example 5 - Effects of Topical Formulations of Gemini Cationic Surfactants in a Murine Model

a) *Treatment groups*

A murine model was used to examine the effects of topical formulations of the gemini cationic surfactant. The animal experiments were approved by the University Committee on Animal Care and Supply Protocol Review Committee. CD1 mice were obtained from the Animal Resource Center, University of Saskatchewan, Saskatoon, SK. The mice were anesthetized with isoflurane and close-shaved the day prior to treatment. For the topically treated groups, the animals were anesthetized with acepromazine 2 mg/kg injected subcutaneously, the shaved area was cleaned with distilled water using sterile gauze and dried. Liposomal and microemulsion formulations (50 μL containing 25 μg pGTmCMV.IFN-GFP plasmid for each animal) were painted on the shaved area, using a pipette. For the groups having several treatments, the application was repeated at 24 and 48 h intervals. Each animal received a total dose of 75μg

pGTmCMV.IFN-GFP plasmid. In the injected groups, the animals were intradermally treated on their backs at three locations, with liposomal plasmid formulation (5μg plasmid/site), low and high concentration of aqueous plasmid solution (2.5 and 5 μg plasmid/site) or phosphate buffered saline (PBS). The total dose for the animals injected with liposomal plasmid formulation was 15 μg pGTmCMV.IFN-GFP/animal and for the injected DNA solutions 7.5 μg plasmid (low dose) and 15 μg plasmid/ animal (high dose). The naive animals were left untreated. Blood samples, skin biopsies and lymph nodes from inguinal and axillary sites were taken 24 h after the last treatment.

b) *Sample processing*

Plasma was separated by centrifugation from blood collected from animals by cardiac puncture, and stored at -20°C. The skin was rinsed with distilled water using sterile gauze and the treated areas were excised. The samples were snap-frozen in liquid nitrogen, and stored at -80°C. The axillary and inguinal lymph nodes were collected, snap-frozen in liquid nitrogen, and stored at -80°C. The skin was homogenized under liquid nitrogen using a biopulverizer. The device was thoroughly cleaned, disinfected with 70% ethanol and dried in a laminar flow hood, under UV light for 15 minutes between the recovery from different groups to avoid cross-contamination. The homogenized skin samples for ELISA and the lymph nodes were resuspended in 500 μL PBS containing leupeptin 10 μg/mL (Sigma) and soybean trypsin inhibitor 20 μg/mL (Sigma). The homogenized skin samples for PCR were resuspended in proteinase K solution (200μg/mL), incubated for 2 hours at 56°C, boiled for 10 minutes, chilled on ice for 10 minutes and centrifuged at 16000*g* at 4°C for 20 minutes. The supernatants were collected and used for PCR.

The lymph nodes were homogenized in microfuge tubes with disposable pellet pestles (Kontes, VWR, Mississauga, ON). The homogenates were vortexed for 1 minute, sonicated for 30 seconds and kept on ice for 1 minute. This cycle was repeated three times. All samples were centrifuged at 16,000*g* for 15 minutes. The supernatants, free of cell debris, were collected and stored at -20°C.

Example 6 - Enzyme-linked immunosorbent assay (ELISA)

ELISAs were performed using round bottom 96-well plates (Immulon II, Dynatech Laboratories, Chantilly, VA). The plates were coated with 50 μL/well of capture antibody, rat anti-mouse IFNγ (Pharmingen, Mississauga, ON) 2 μg/mL coating buffer and incubated for 24 hours at 4°C. The wells were blocked with 1% bovine serum albumin (BSA) (New England Biolabs, Mississauga, ON) solution in PBS at room temperature for one hour. IFNγ standard (Pharmingen) of 250-2000 pg/mL concentration and was used in 1% BSA solution on plates. PBS was used as a blank control. The supernatants from cell cultures and the skin and lymph homogenates, as well as the serum from mice, were diluted one in four on the plates. They were incubated overnight at 4°C. Biotinylated rat anti-mouse IFNγ (Pharmingen) was added at 0.5 ng/mL concentration in 1% BSA solution. The plates were incubated for a further 2 h at room temperature. The streptavidin-alkaline phosphatase conjugate (Jackson Immuno Research Laboratories, Inc., West Grove, PA) was added in 1:5000 dilution and incubated for 1 h at room temperature, followed by addition of 4-nitrophenyl phosphate di(tris) salt 1 mg/mL in PNPP buffer (1% diethanolamine, 0.5mM $MgCl_2$, pH9.8) (Sigma). Optical density of the samples was measured at 405nm using a Benchmark Microplate Reader (BioRad, Mississauga, ON). The concentration of the IFNγ vas calculated from the standard IFNγ curve, using recombinant murine IFNγ (Pharmingen).

Example 7 - Antiviral Assay for Testing the Activity of IFNγ

The method for antiviral assay was provided by Dr. C. Havele (Department of Microbiology and Immunology, University of Saskatchewan, SK). Murine IFNγ was tested for biological activity based on the reduction of the viral cytopathic effect. L929 cells (ATTC# CCL-1) were plated on a 96-well flat bottom plate (Greiner Labortechnik GmbH, Germany) ($5x10^4$ cells/well) in RPMI-1640 medium supplemented with 10% FBS, 0.1mM 2-mercaptoethanol (Sigma), 0.8mM sodium pyruvate (Sigma) and Antibiotic Antimycotic Solution (Sigma). Supernatants from PAM212 cells transfected with pGTmCMV.IFN-GFP and containing expressed IFNγ were added to the wells in serial dilution. The plates were incubated for 24 h at 37°C, 5% CO_2. The media were replaced with a 100-fold greater titer of endomyocarditis (EMC) virus

(Familletti *et al.*, 1981) in 100μL media, and incubated overnight at 37°C, 5% CO_2. The wells were washed with PBS and the cells fixed for 15 minutes in 4% formaldehyde (Sigma) and stained with 0.05% crystal violet (Sigma) solution in 20% methanol for 15 minutes. The plates were then washed and dried. Before measurements were carried out, 100 μL methanol/well was added to the plates. The absorbance was read at 595nm on an automated plate reader (PowerWave$_x$, Biotech Instruments Inc., Winooski, VT). To verify the non-specific antiviral assay, monoclonal antibody against IFNγ activity XMG1.2 (Cherwinski *et al.*, 1987) was added to the supernatants. Recombinant murine IFNγ standard (Pharmingen) was also included in the assay.

Example 8 - Fluorescent and light microscopy

Cells were grown in 24-well plates on cover slips and transfected with plasmids, as described. Twenty four h after transfection, the cells were washed twice with PBS and cover slips mounted. Skin samples snap-frozen in liquid nitrogen were embedded in Tissue-Tek O.C.T. Compound (Canemco, St. Laurent, QB), and cut in 7-μm thick sections. They were mounted on poly-D-lysine-coated microscope slides. Phase contrast and fluorescent images were registered using Axiovert 200M inverted microscope (Zeiss, Germany), with LD-A Plan 40X objective lens. The excitation wavelength for GFP was 488nm and emission wavelength 507nm (FITC filter). Autofluorescence was detected with rhodamine filter (excitation at 570 nm, emission at 590nm).

Example 9 - Fluorescence-activated cell sorter (FACS)

PAM212 cells were seeded on 6-well plates (Costar, Corning NY) at a density of 1 x 10^6 cells/well density and grown to 60-80% confluency. The supplemented MEM was changed to MEM one h prior to transfection. The transfection mixes were prepared with 1μg pGTmCMV.IFN-GFP, using the 16-3-16 gemini cationic surfactant and DOPE liposomes at plasmid DNA: gemini cationic surfactant charge ratio of 1:5, 1:10, 1:20 or 1:40, as described earlier, keeping the concentration of the reagents constant. The supernatants were collected and stored at -20°C. The cells were detached using Versene solution containing 0.05% trypsin (Sigma), pelleted at 4°C and 1000*g* for 5 minutes, washed twice with PBS and resuspended in Fa-

cola (10 mM PBS pH 7.2, 0.2% gelatin, 0.03% sodium azide). The cell sorter was calibrated with non-transfected cells and 10^4 cells of each sample were counted.

Example 10 - Circular Dichroism

Aqueous gemini cationic surfactant solutions and DOPE liposomes were prepared as for *in vitro* transfection and degassed at 37°C in a bath sonicator. Plasmid pGTmCMV.IFN-GFP (20 μg/mL) and gemini cationic surfactant were mixed at DNA:gemini cationic surfactant charge ratio of 1:10, using water or DOPE liposomes as a vehicle. The samples were incubated for 10 min at room temperature prior to measurement. Spectra were recorded by using an Applied Photo Physics π* 180 instrument (Leatherhead, UK) with a 1-nm slit, at 37°C.

Example 11 - Polymerase chain reaction (PCR)

Four primers were designed for nested PCR, amplifying a fragment from the pGTmCMV backbone. The external primers were: sense (pKanEF) 5-ACT CAC CGA GGC AGT TCC AT-3' (SEQ ID NO: 1) and antisense (pKanER) 5'-GGT AGC GTT GCC AAT GAT GT-3'(SEQ ID NO: 2), amplifying a 540-bp fragment of the pGTmCMV.IFN-GFP plasmid. The internal primers were: sense (pKanIF) 5'-ATG GCA AGA TCC TGG TAT CG-3' (SEQ ID NO: 3) and antisense (pKanIR) 5'-TTA TGC CTC TTC CGA CCA TC-3' (SEQ ID NO: 4), which amplifies a 459-bp fragment from the previous reaction. Standard dilutions were prepared with the pGTmCMV.IFN-GFP plasmid at 10^2, 10^3, 10^4, 10^5 and 10^6 copies/PCR reaction. The PCR mixes were prepared according to the manufacturer's protocol. All primers and PCR Reagent System were purchased from Invitrogen Life Technologies, Carlsbad, CA. Thirty five μL of supernatant obtained from each skin homogenate was used for amplification with the external primers, and 2μL of the PCR product was used for amplification with the internal primers. Techne Genius unit (Techne Incorporated, Princeton, NJ) was used, under the following conditions: hot start for 4 m at 94°C, denaturation at 94°C for 45 s, annealing at 56°C for 30 s, extension for 30 s at 72°C and final extension at 72°C for 7 m. The reaction was carried out in thirty cycles. The PCR products were run in 1% agarose gel, the bands stained with ethidium bromide and quantified, based on standard dilutions.

Example 12 - Statistical Analyses

Statistical analyses included ANOVA and Kruskal-Wallis test using SPSS 11.5 for Windows (SPSS Inc., 233 S. Wacker Drive, 11th floor Chicago, Illinois 60606).

Results

i. Plasmid constructs and testing of expression in PAM212 cells

The expression of the *IFNγ* gene from two plasmid constructs was tested in murine PAM 212 keratinocyte cell culture. ELISA test of the cell supernatants showed high expression of IFNγ 24h after transfection with the pGTmCMV.IFN-GFP plasmid, whereas significantly lower IFNγ expression was observed with the plasmid based on pIRES backbone (pIRES.IFN-GFP) (Figure 2). Significant differences were found between the IFNγ expression of pIRES.IFN-GFP and pGTmCMV.IFN-GFP (ANOVA $p<0.05$). After dosing the cells for 24 h, the expression was at a maximum, and by 48 h, it decreased to about 16% of the 24h level.

The IFNγ secreted into the supernatants was biologically active as demonstrated by antiviral assay (Example 7). The average IFNγ concentration was 119.91 ±39.62 ng/5 x 10^5 PAM 212 cells. Preincubation of IFNγ containing supernatants with the XMG1.2 monoclonal antibody against IFNγ resulted in the abolition of cytoprotection. Supernatants from cells incubated with the null (non-coding) plasmids of either backbone did not contain IFNγ (Figure 2) nor did they show any antiviral activity (results not shown).

The GFP expression followed the pattern of IFNγ expression with the cells transfected with pGTmCMV.IFN-GFP showing stronger fluorescence than those transfected with pIRES.IFN-GFP (data not shown). The cells treated with null plasmids did not fluoresce. The merged images of cells transfected with pGTmCMV.IFN-GFP and pGTmCMV are shown in Figure 3. GFP expression can be seen only in the cells transfected with the plasmid containing the *GFP* gene. The micrographs were overlays of phase contrast on fluorescent images.

ii. Optimization of cationic gemini cationic surfactant – DNA ratios and transfection duration

The lipid formulations were optimized for plasmid DNA: gemini cationic surfactant charge ratio using the 16-3-16 compound. The optimal plasmid DNA: gemini cationic surfactant charge ratio was determined by comparing the quantity of expressed IFNγ with the number of fluorescent cells and determining cell viability. Figure 4 is a graph showing IFNγ expression in PAM 212 keratinocytes assessed by ELISA, GFP expression and cell viability by FACS. The cells were transfected with either transfection mixtures of DOPE and the 16-3-16 gemini cationic surfactant at plasmid DNA: gemini cationic surfactant charge ratio of 1:5, 1:10, 1:20 or 1:40, or Lipofectamine Plus™ Reagent, using 0.2μg pGTmCMV.IFN-GFP. The percentage of viable and fluorescent cells is represented on the secondary Y axis.

PAM 212 cells, transfected with 0.2μg pGTmCMV.IFN-GFP using gemini cationic surfactant and DOPE at DNA: gemini cationic surfactant charge ratios of 1:5, 1:10, 1:20 and 1:40, showed optimum transfection at plasmid DNA: gemini cationic surfactant charge ratio of 1:10. At this charge ratio IFNγ level reached approximately 10.78ng/10^6 cells with 70% cell viability, whereas the commercially available Lipofectamine Plus™ Reagent (DOSPA:DOPE 3:1; at the concentration recommended by the manufacturer) produced about 17.79 ng/10^6 cells IFNγ (1/40 diluted supernatant) and about 28% cell viability. Increase in the cationic charge ratio with gemini cationic surfactants resulted in lower cell viability and lower protein expression. At plasmid DNA: gemini cationic surfactant charge ratio of 1:40 cell viability fell to 20% and no IFNγ expression could be detected. GFP expression showed a similar trend to IFNγ expression as shown by the fluorescence curve.

Figure 5 is a graph showing the influence of transfection duration on the efficiency of the transfection. PAM 212 cells were transfected with 0.2μg pGTmCMV.IFN-GFP using the transfection mixtures consisting of DOPE and the 16-3-16 gemini cationic surfactant at plasmid DNA: gemini cationic surfactant charge ratio of 1:10 for 6, 8, 10 or 24 hours. The optimum duration of transfection was found to be 24h.

iii. Effect of gemini cationic surfactant series 12-n-12 and a 16-3-16 on the transfection of PAM212 cells with pGTmCMV.IFN-GFP plasmid

Eight different gemini cationic surfactants (plasmid DNA-gemini cationic surfactant-DOPE; plasmid:gemini cationic surfactant charge ratio 1:10) were tested to determine the effect of head group spacer length and alkyl chain length on their transfection ability. The transfection efficiency was found to be dependent on the length of the spacer between the two positively charged head groups, with the C3 spacer showing the highest activity (both 12-3-12 and 16-3-16) (Table 1).

Table 1. The magnitude of *in vitro* transfection correlated with physico-chemical characteristics of the gemini cationic surfactants.

Gemini compound	IFN-γ expression [ng/5 x 10^5 cells]	Physico-chemical parameters				
		CMC[a] [mM]	a[a] [nm^2/molecule]	α[a]	Γ[b] x 10^6 [mol/m^2]	Krafft point[c] [°C]
12-3-12	1.11 ± 0.65	0.98 ± 0.04	0.98	0.23 ± 0.02	1.58	12.7
12-4-12	0.66 ± 0.32	1.17 ± 0.04	1.10	0.26 ± 0.02	1.43	10.6
12-6-12	0.48 ± 0.17	1.09 ± 0.04	1.40	0.34 ± 0.02	1.16	<0
12-8-12	0.24 ± 0.12	0.84 ± 0.03	1.78	0.46 ± 0.04	0.94	<0
12-10-12	0.29 ± 0.10	0.62 ± 0.03	2.16	0.51 ± 0.06	0.75	<0
12-12-12	0.33 ± 0.16	0.36 ± 0.03	2.14	0.56 ± 0.08	0.73	13.6
12-16-12	0.49 ± 0.19	0.12 ± 0.01	1.44	0.59 ± 0.08	0.83	24.3
16-3-16	1.63 ± 1.37	0.026 ± 0.001	1.02	0.35 ± 0.02	1.08	42

CMC (critical micellar concentration); α (degree of micelle ionization); a (head group areas based on activity); Γ (surface excess concentration); [a]from Wettig *et al.*, 2001, [b]from Alami *et al.*, 1993, [c]from Zana, 2002.

In the 12-n-12 series gemini cationic surfactants, a hyperbolic pattern is noticeable with minimum expression of protein occurring at spacer C8. Significantly higher IFNγ levels were found when the cells were transfected with 12-3-12, 12-4-12 and 16-3-16 versus the other compounds with longer linkers ($p < 0.001$). No IFNγ was detected when the cells were transfected with DOPE or gemini cationic surfactant, alone, nor with plasmid without transfection agents.

Circular dichroism (CD) indicated structural changes in the DNA structure, induced by the gemini cationic surfactant/DOPE liposomes. Figure 6A illustrates the circular dichroism spectra of plasmid-gemini-DOPE liposomal complexes, showing circular dichroism spectra of the pGTmCMV.IFN-GFP plasmid 20μg/mL in water, DNA-12-3-12-DOPE liposomes or DNA-12-16-12-DOPE liposomes, and DNA-16-3-16-DOPE liposomes. Figure 6B illustrates the circular dichroism spectra of plasmid-gemini complexes, showing circular dichroism spectra of the pGTmCMV.IFN-GFP plasmid 20μg/mL in water, in DOPE suspension or coupled with 16-3-13 at plasmid DNA: gemini cationic surfactant charge ratio of 1:10 in water, and in DOPE suspension.

CD spectra show that the gemini cationic surfactant/DOPE liposomes decrease the positive peak at 290 nm and shift the 260-nm peak to negative values (Figure 6A). Although the gemini cationic surfactants or DOPE, alone, or gemini cationic surfactant/DOPE liposomes induced a shift of the 260-nm peak to negative values in the spectrum of DNA, the peak at 290 nm was only decreased by the gemini cationic surfactant/DOPE liposomes (Figure 6B). The pattern changes induced by the 12-carbon series was similar, showing a peak (in the negative region) at 230-240nm. This peak was higher for the 16 carbon spacer than the 3-carbon spacer. Interestingly, the peak at 230 nm disappeared for the 16-3-16 compound (Figure 6A).

iv. Topical transfection of pGTmCMV.IFN-GFP plasmid using gemini cationic surfactant (16-3-16)- lipid systems

Topical formulations were prepared as detailed in Example 4, where two topical delivery systems, namely a cationic liposome formulation and a cationic microemulsion, were prepared with the gemini 16-3-16 surfactant. The formulations were characterized by visual appearance, microscopy and pH. The effects of such formulations were investigated using a murine model (Example 5). Blood samples, skin biopsies and lymph nodes from inguinal and axillary sites were obtained for assessment following treatment.

Figure 7A is a graph showing IFNγ expression in skin in the mice treated with the pGTmCMV.IFN-GFP plasmid and gemini lipid 16-3-16 in various formulations. Results are expressed as amount of IFNγ/cm^2 treated skin for the topical treatment and as amount of IFNγ/animal for the injected groups. Figure 7B is a graph showing IFNγ expression in lymph nodes in the mice treated with the pGTmCMV.IFN-GFP plasmid and gemini lipid 16-3-16 in various formulations. Results are expressed as amount of IFNγ/animal for all groups. The groups and treatments are set out below:

Group	Treatment
DNA-top(3x24h)	topically treated with pGTmCMV.IFN-GFP 25 μg in 50μL in aqueous solution for three days (total dose 75 μg)
DNA-L-top(24h)	topically treated with pGTmCMV.IFN-GFP in cationic gemini liposomal formulation 25 μg in 50μL for one day (total dose 25 μg)
DNA-L-top(3x24h)	topically treated with pGTmCMV.IFN-GFP in cationic gemini liposomal formulation 25 μg in 50μL for three days (total dose 75 μg)
DNA-ME-top(3x24h)	topically treated with pGTmCMV.IFN-GFP in cationic gemini microemulsion formulation 25 μg in 50μL for three days (total dose 75 μg)
DNA-L-inj(24h)	intradermally injected with pGTmCMV.IFN-GFP in cationic gemini liposomal formulation 5 μg in 10μL (total dose 15 μg) for one day
DNA-inj-h(24h)	intradermally injected with pGTmCMV.IFN-GFP in aqueous solution (high concentration - 5 μg in 10μL, total dose 15 μg) for one day
DNA-inj-l(24h)	intradermally injected with pGTmCMV.IFN-GFP in aqueous solution (low concentration – 2.5 μg in 10 μL, total dose 7.5 μg) for one day
PBS-inj(24h)	intradermally injected with 10 μL PBS for one day
L-top(3x24h)	topically treated with 50 μL control cationic gemini liposomal (no DNA) formulation for three days
naive	untreated

Significant differences were observed at the $p<0.05$ level (ANOVA). Topical treatment of mice with pGTmCMV.IFN-GFP plasmid in liposomal or microemulsion lipid formulations resulted in high levels of IFNγ expression in the skin (Figure 7A) and lymph nodes (Figure 7B). Three times application (24h apart) with gemini cationic liposomes (75μg DNA total dose) led to higher IFNγ expression in the skin than a single application (25 μg/dose), both in the skin (about 20% higher) (Figure 7A) and the lymph nodes (about 40% higher) (Figure 7B). Repeated application of liposomal or microemulsion formulations induced approximately 50% higher expression than three times application of DNA solution in the skin. Gemini cationic liposome and gemini microemulsion (75μg DNA total dose) produced similarly high levels of IFNγ in the skin (811 vs 934 pg/cm^2 skin). However, the gemini cationic liposomes produced higher IFNγ expression in the lymph nodes (1421 vs 873 pg/animal). The IFNγ levels in the skin of these groups were statistically different from the naïve group ($p<0.01$). The IFNγ expression after a single topical application of the plasmid in gemini liposomes (25μg/dose) was similar to the intradermal injection of naked DNA solution at 15μg DNA/dose. No IFNγ could be detected in the serum obtained from the animals in any of the groups.

Figures 8A-F are color photographs of a fluorescence microscopic evaluation of the GFP expression in skin of mice treated with the pGTmCMV.IFN-GFP plasmid and gemini lipid 16-3-16 in various formulations (Examples 8 and 9). Phase contrast micrographs were overlaid on fluorescent micrographs of 7-μm thick skin sections. Figures 8A-F are color photographs of a fluorescence microscopic evaluation of the GFP expression in skin of mice treated with the pGTmCMV.IFN-GFP plasmid and gemini lipid 16-3-16 in various formulations. Figures 8A-D show skin obtained from mice topically treated with pGTmCMV.IFN-GFP in cationic gemini liposomal formulation 25 μg in 50μL for three days (total dose 75 μg) (DNA-L-top(3x24h)). Figure 8E shows skin obtained from mice intradermally injected with pGTmCMV.IFN-GFP in aqueous solution (low concentration of 2.5 μg in 10 μL, total dose 7.5 μg) for one day (DNA-inj-l(24h)). Figure 8F shows skin obtained from mice topically treated with 50 μL control cationic gemini liposomal formulation for three days (L-top(3x24h)).

GFP expression was detected in the skin treated with liposomal formulation in the epidermis (Figures 8A-D) and around the injection site in skin injected with 2.5μg DNA/site (Figure 8E). No GFP fluorescence was observed in the skin of animals treated with control (no DNA) liposomal formulation (Figure 8F). Autofluorescence was ruled out by comparing the images to those taken in the rhodamine emission band. Plasmid could be detected by nested PCR in the skin of the animals treated intradermally and topically with the gemini cationic liposomal formulation (Table 2). The ethidium bromide-stained bands were quantified based on standard dilutions of the same plasmid.

Table 2. Nested PCR for pGTmCMV.IFN-GFP plasmid detection in the skin.

Treatment	# copies of pGTmCMV.IFN-GFP/mg skin
DNA-L-top (1x24h) (n=4)	0.86 ± 0.72 x 10
DNA-L-top (3x24h) (n=4)	0.52 ± 0.4 x 10
DNA-ME-top (3x24h) (n=4)	0.07 ± 0.01 x 10
DNA-L-inj(1x24h) (n=3)	17.86 ± 3 x 10
DNA-inj-h (1x24h) (n=4)	0
PBS-inj (1x24h) (n=3)	0

Approximately the same number of plasmid copies were detected in the skin of the animals after single or three times treatments (17.21 x 10^4 copies/mg skin vs. 10.39 x 10^4 copies/mg skin) and 20-30 fold higher levels in the animals injected with gemini cationic liposomal formulation of 5μg DNA/site (357.27 x 10^4 copies/mg skin). Low plasmid level was detected in the skin of the gemini microemulsion treated group. No plasmid was present in the skin of animals injected with 5μg DNA solution/site, nor in the control group.

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All publications mentioned in this specification are indicative of the level of skill in the art to which this invention pertains. To the extent they are consistent herewith, all publications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference. No admission is made that any cited reference constitutes prior art.

Although the foregoing invention has been described in some detail by way of illustration and example, for purposes of clarity and understanding it will be understood that certain changes and modifications may be made without departing from the scope or spirit of the invention.

ABSTRACT

The invention provides a delivery system comprising a gemini surfactant with a biologically active agent for administration to the skin or mucosal membrane for treatment of skin disorders and metabolic diseases. The invention also provides pharmaceutical compositions comprising the delivery system as set out above, in admixture with one or more pharmaceutically acceptable carriers, for the treatment of skin disorders and metabolic diseases. Methods of use and preparation are also described.

FIG. 1A

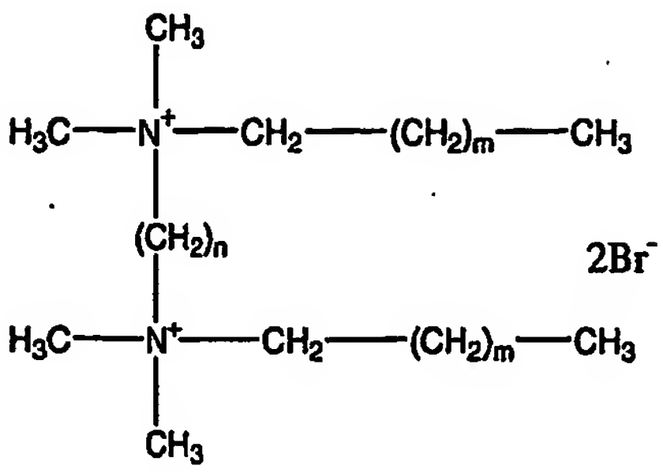

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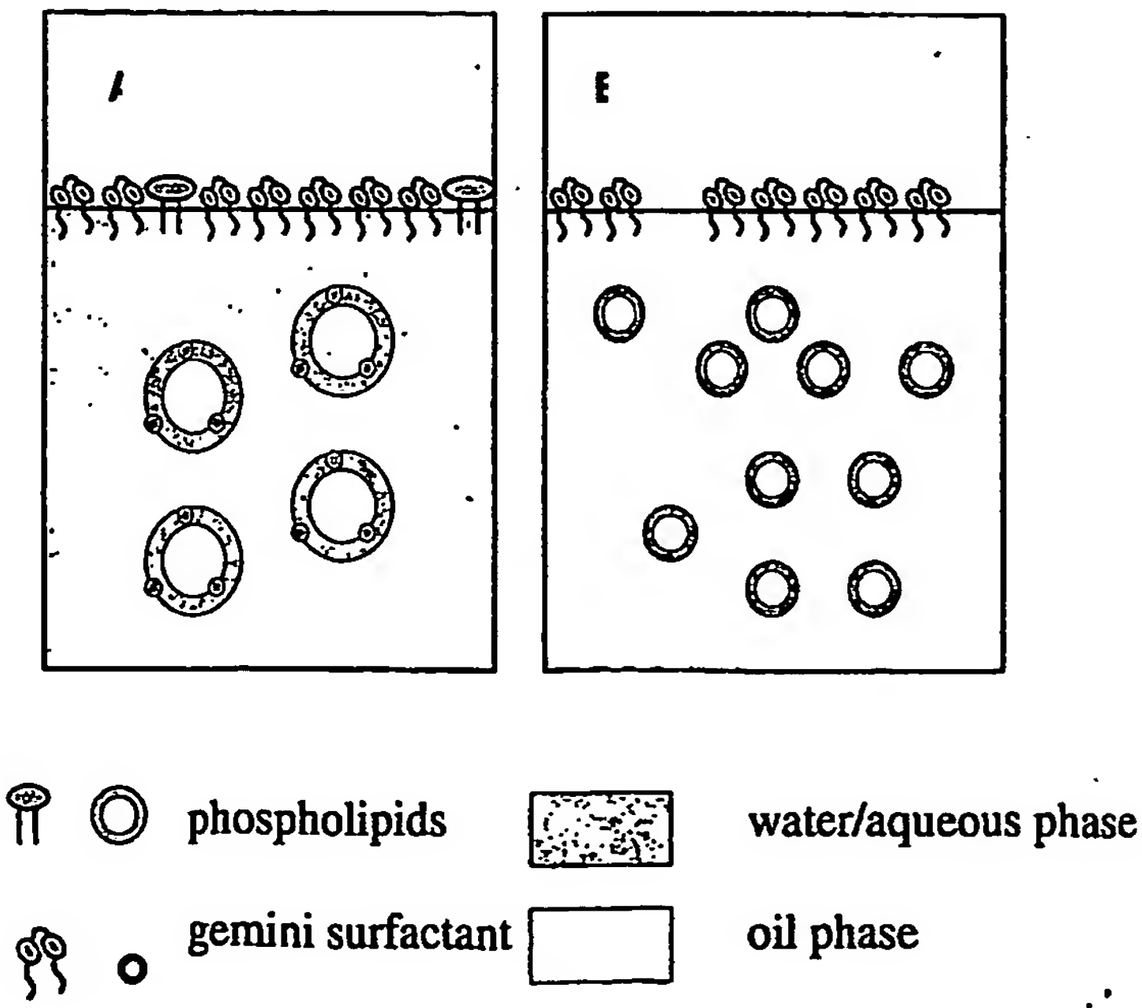
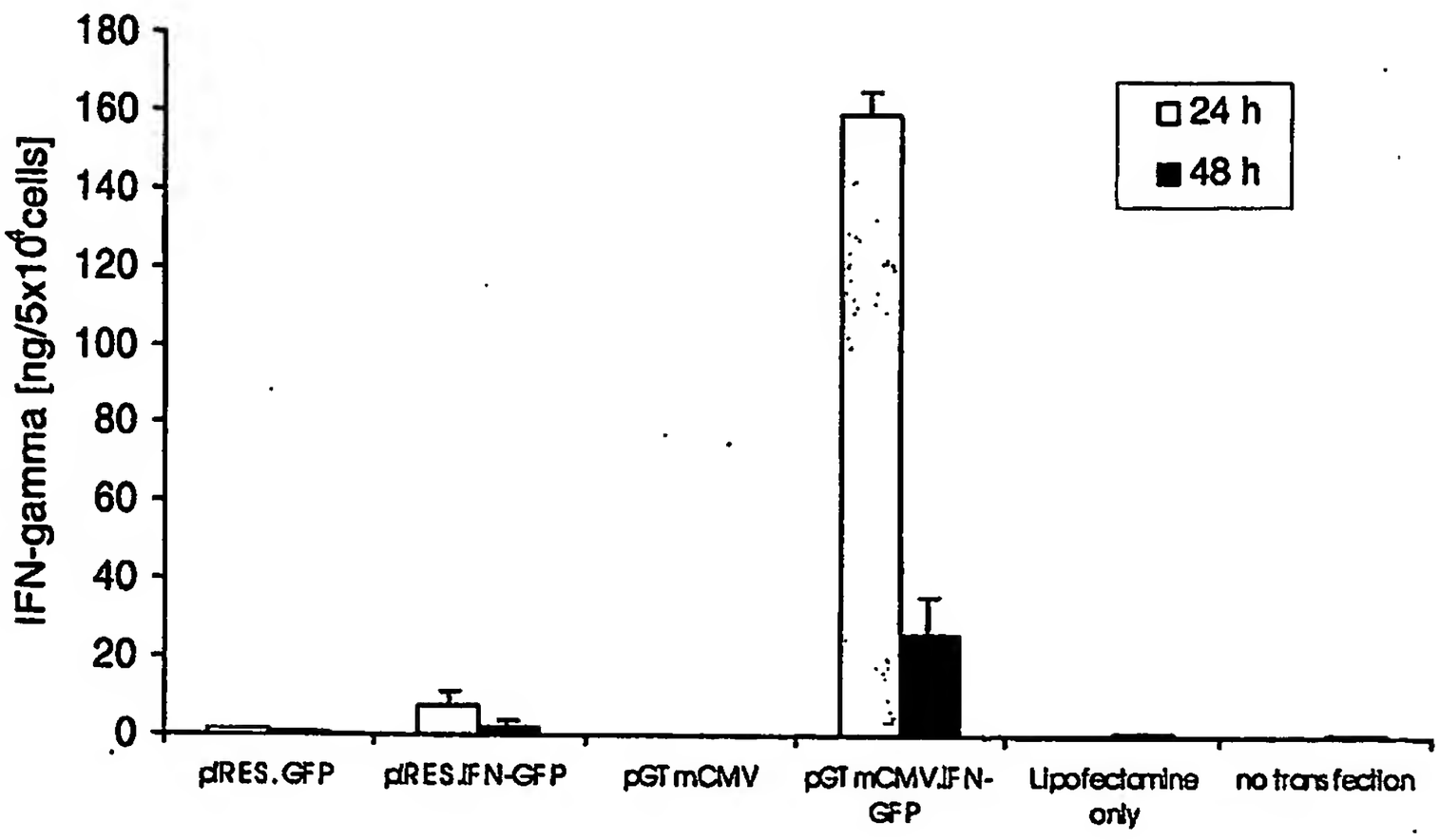
FIG. 1B

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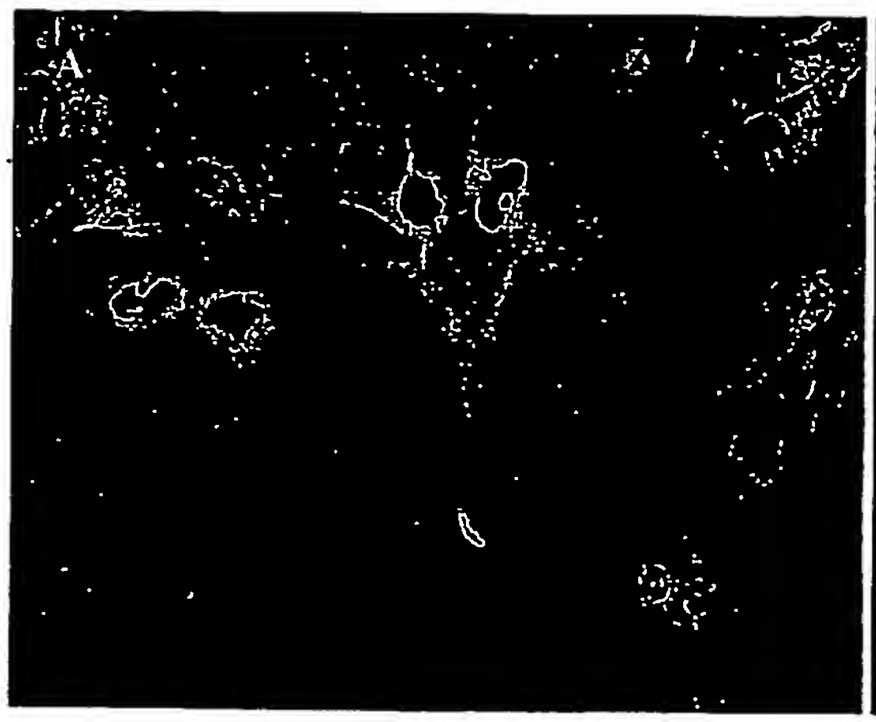

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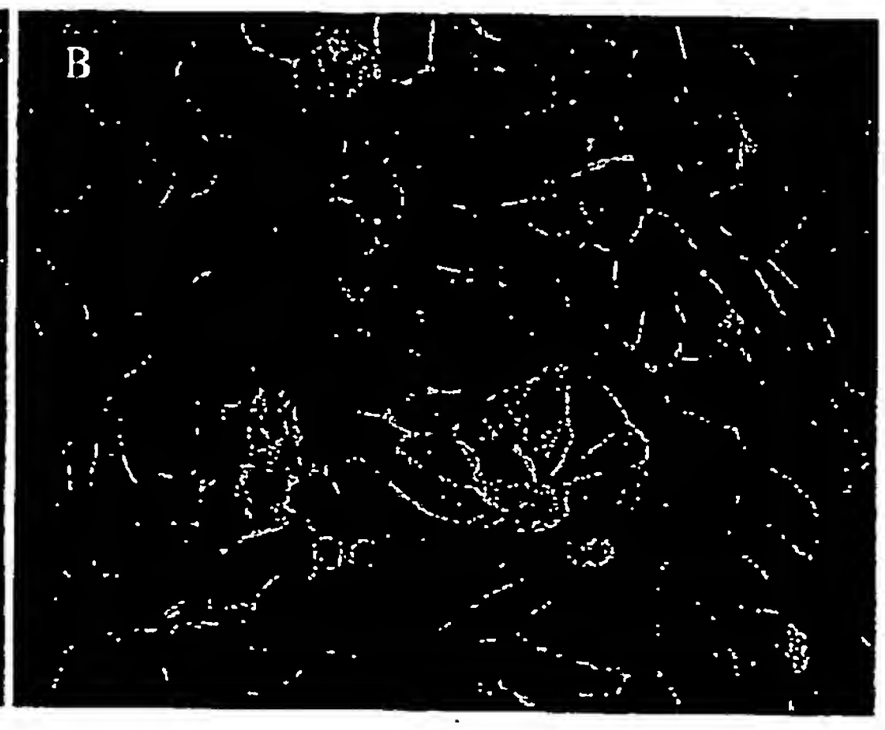

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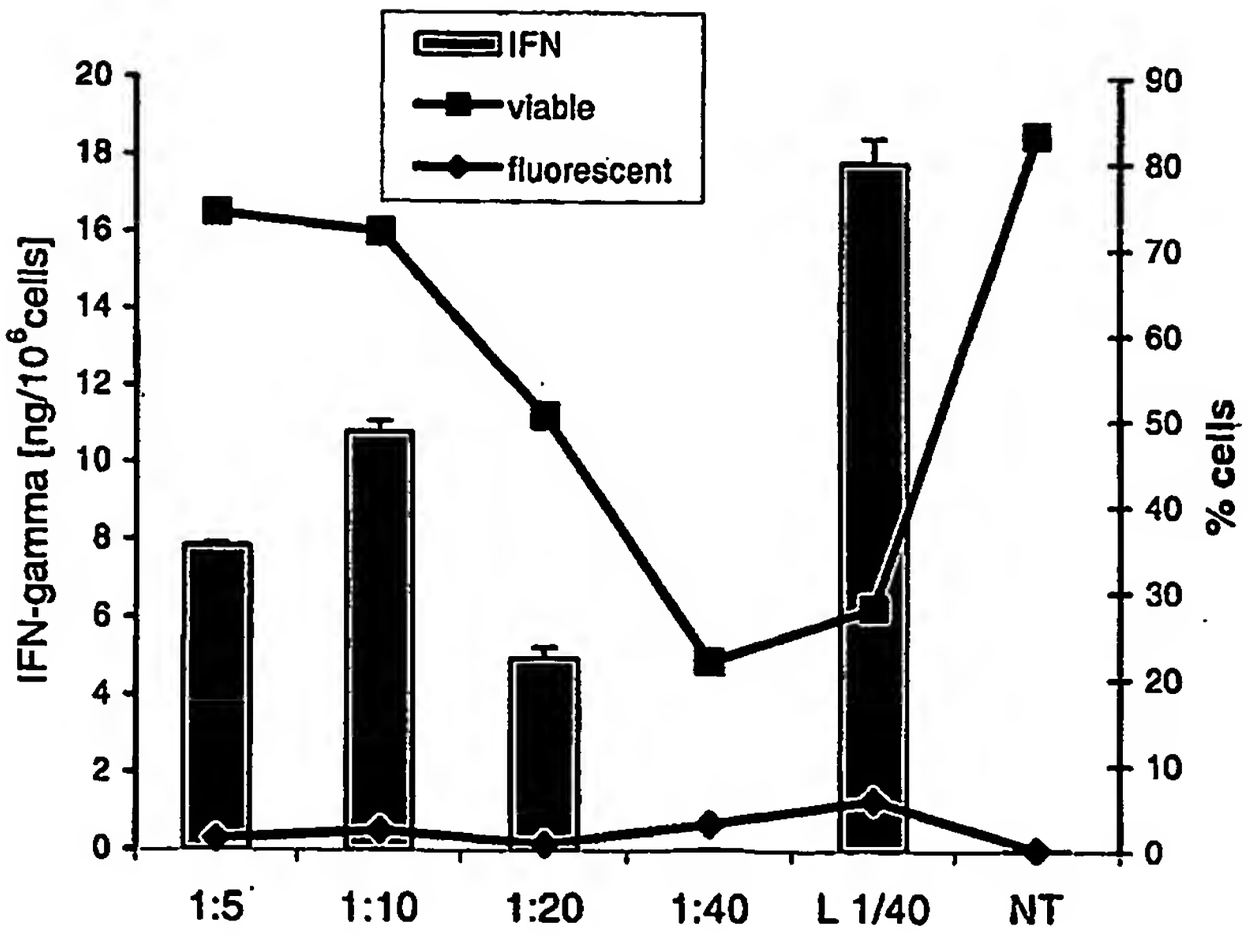
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FIG. 4

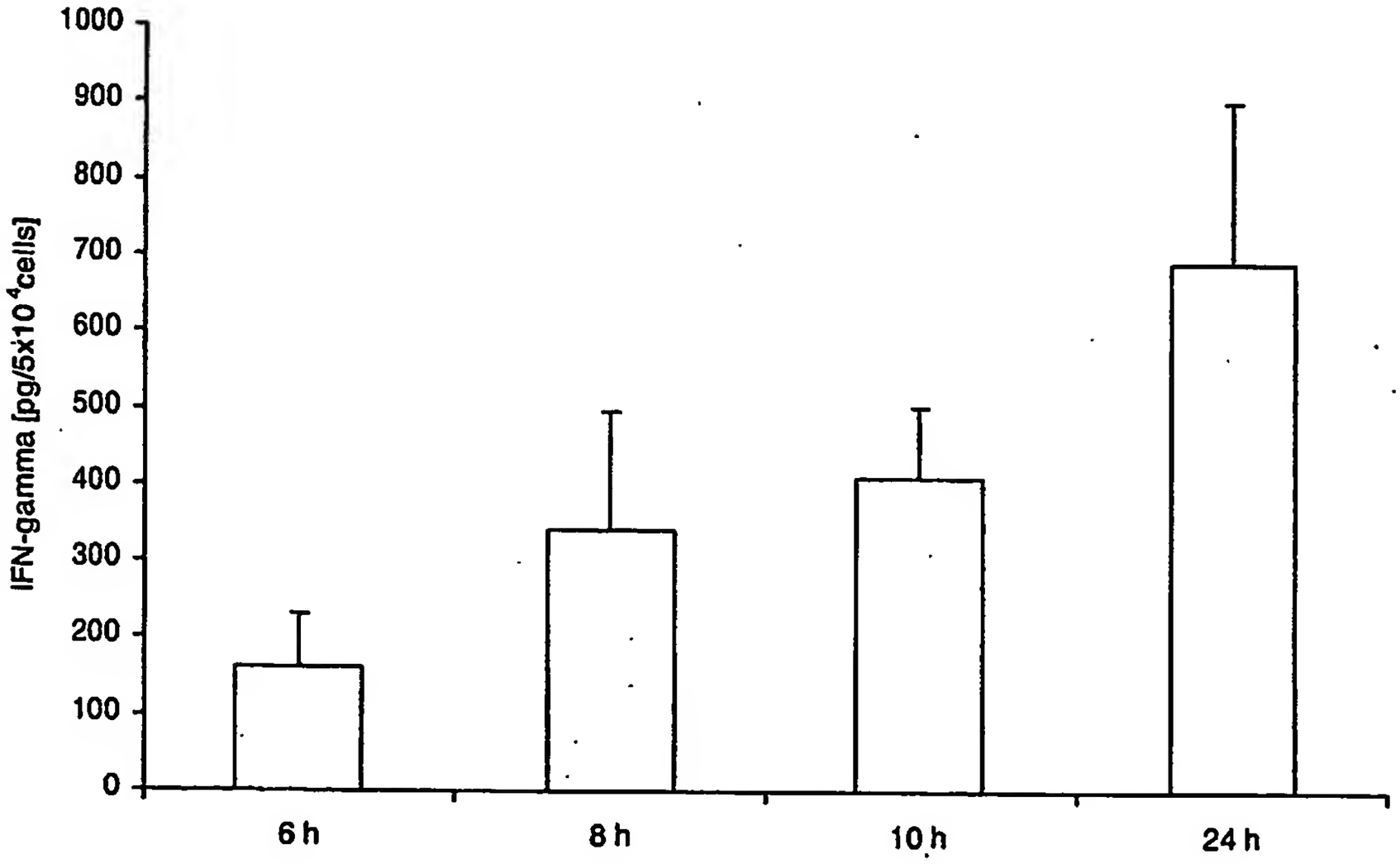
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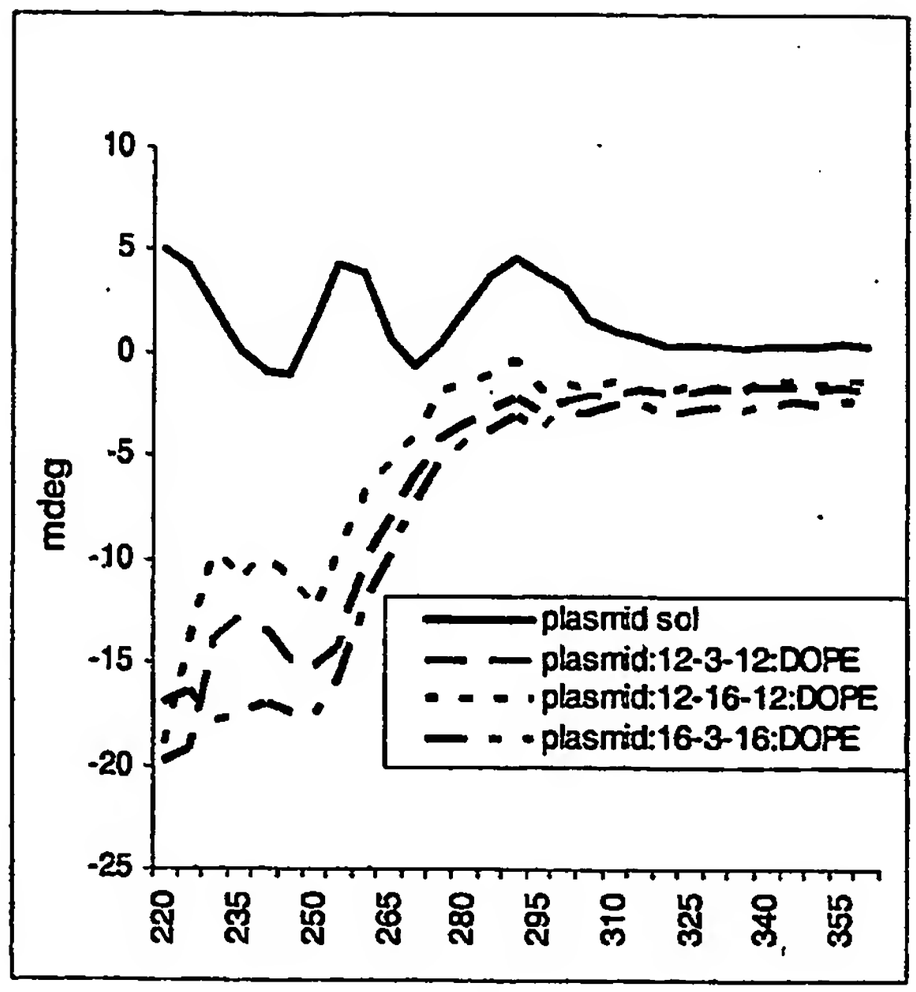
FIG. 5

FIG. 6A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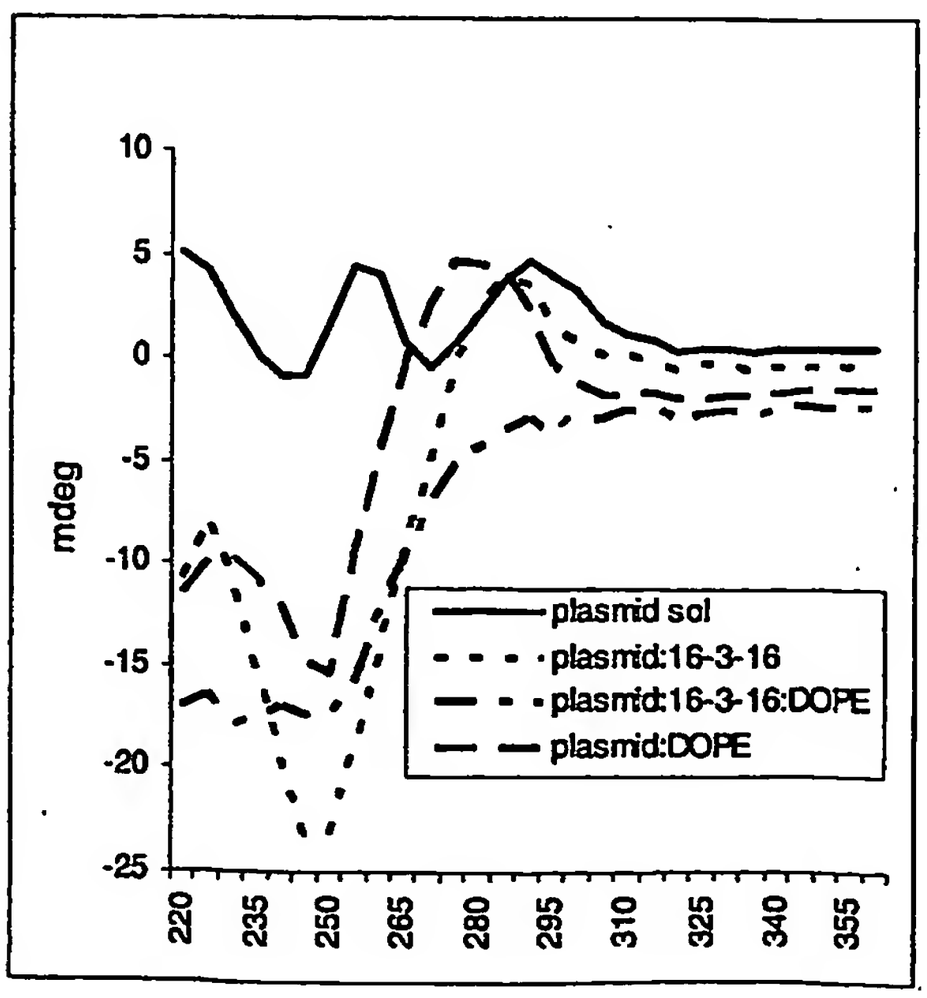
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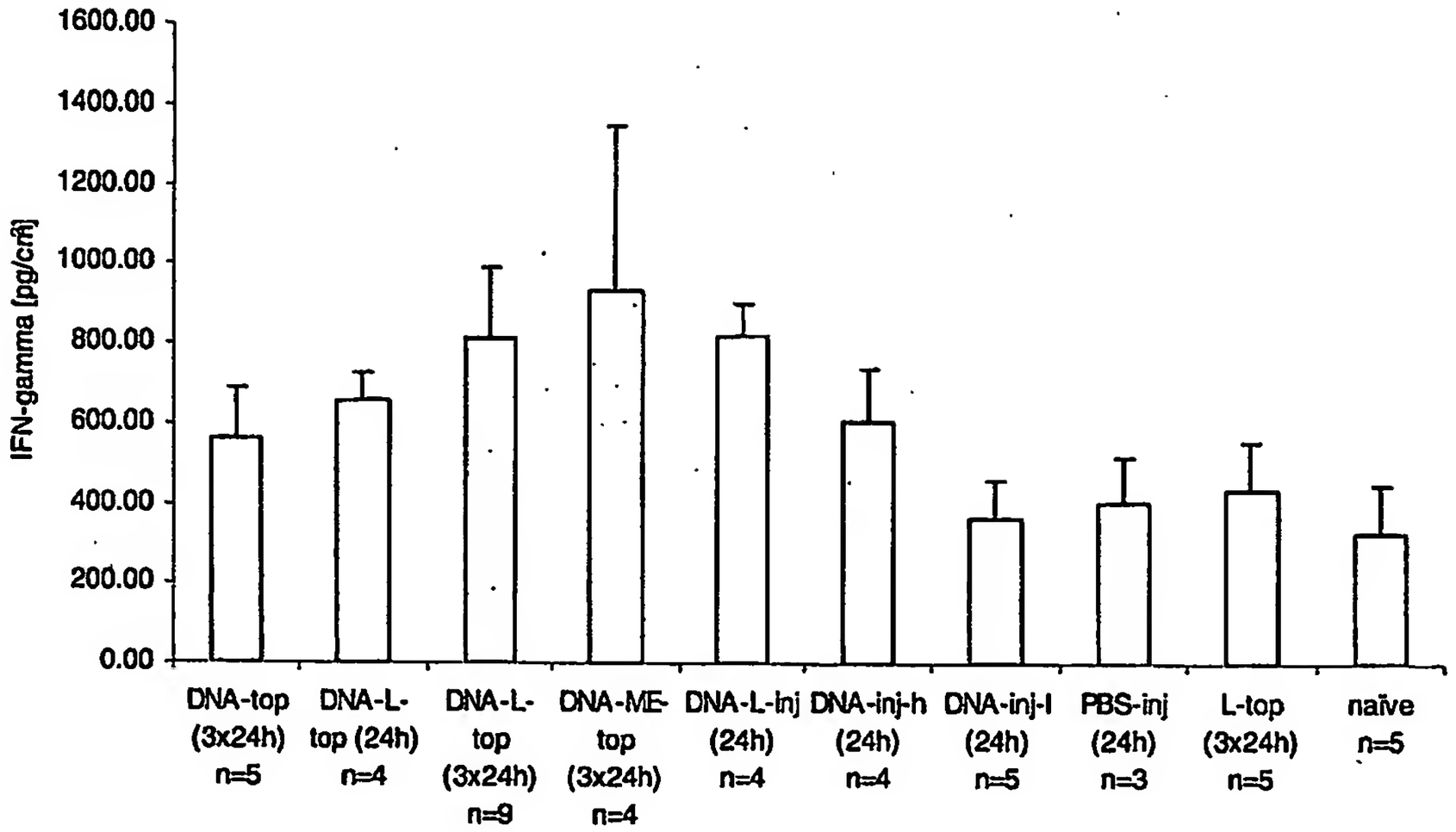
FIG. 7A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DNA-L-top (3x24h) n=9
DNA-ME-top (3x24h) n=4
DNA-L-inj (24h) n=4
DNA-inj-h (24h) n=4
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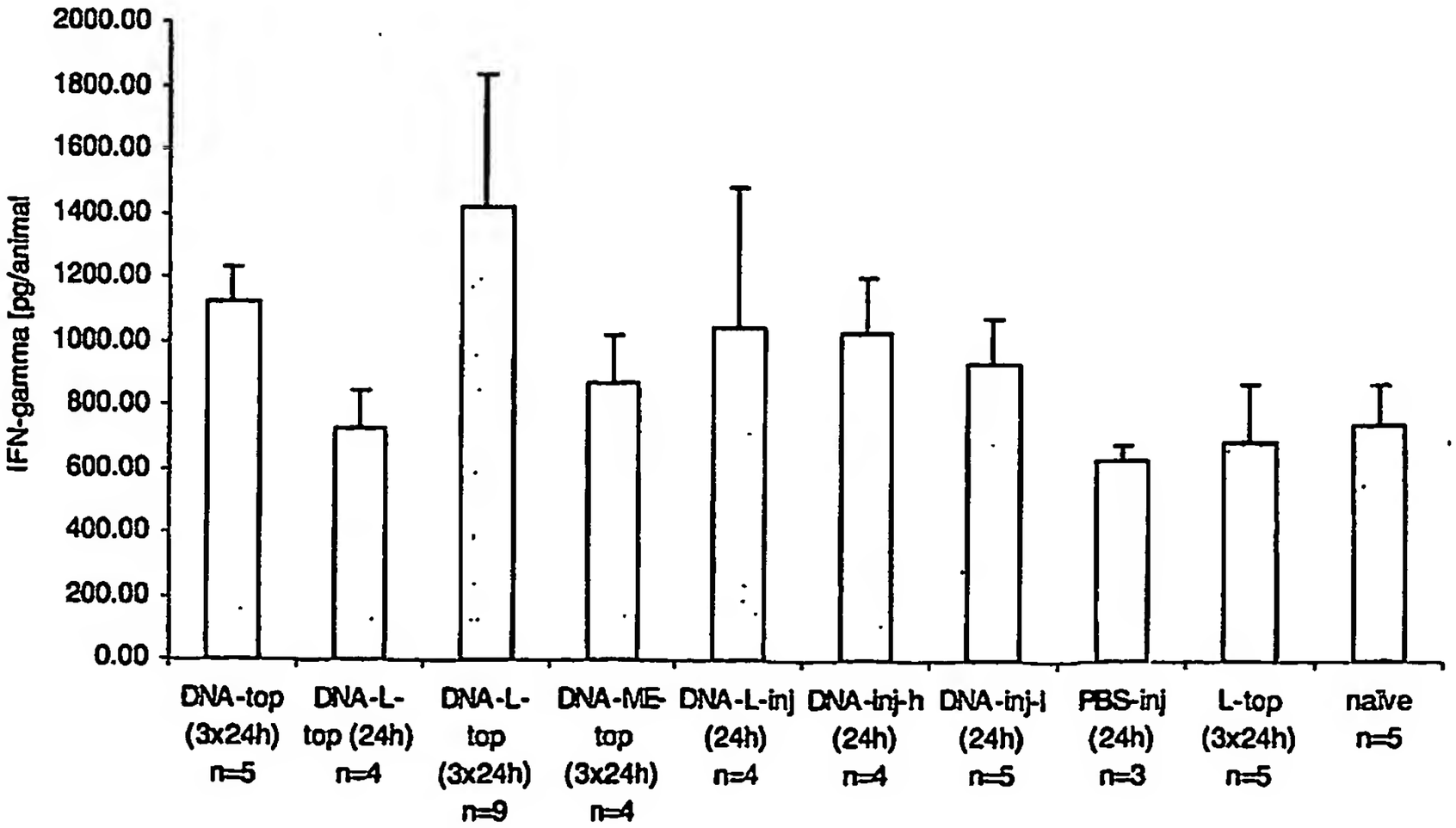
L-top (3x24h) n=5
naïve n=5

FIG. 7B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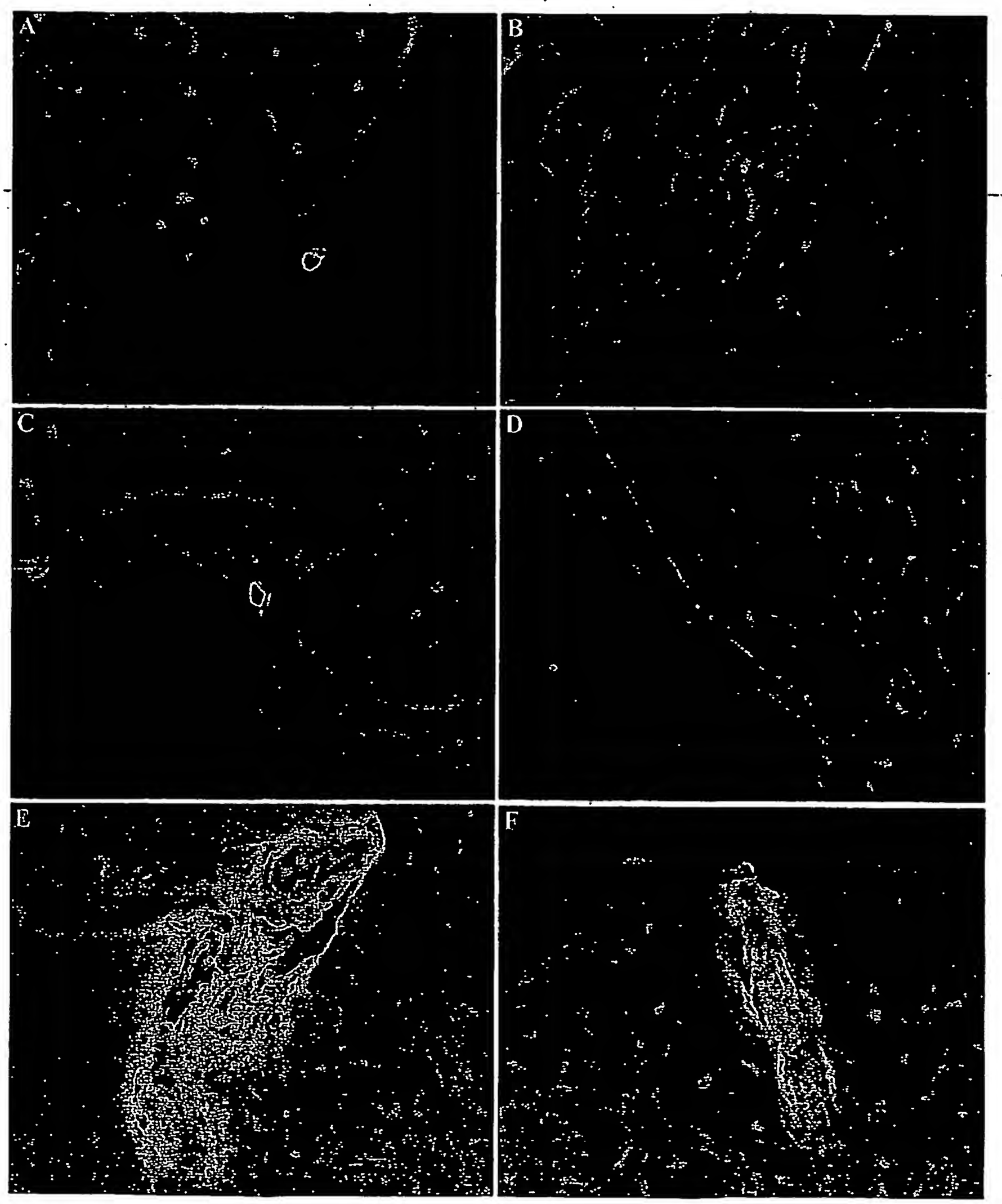

FIG. 8

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